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Cytotoxicity of Tamoxifen-Loaded Solid Lipid Nanoparticles

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1. Introduction

Breast cancer is one the most important health concerns of the modern society (Ferlay *et al.,* 2007). Worldwide, it is estimated that over one million new cases of breast cancer are diagnosed every year, and more than 400 thousands will die from the breast cancer (Coughlin & Ekwueme, 2009) The life-time risk in women contracting breast cancers is estimated to be 1 in 8, which is the highest among all forms of cancers. (DevCan, 2004). Although the mortality rates from breast cancers have decreased in most developed countries because more frequent mammographic screening and extensive use of tamoxifen, it still remains the second highest in women (Clark, 2008). Breast cancer incidence rates were reported to have doubled or tripled in developing countries in the past 40 years (Anderson *et al.,* 2008; Porter, 2008). The main options for breast cancer treatment include surgery, radiation therapy and chemotherapy (Mirshahidi & Abraham, 2004). Surgical procedures usually lead to significant morbidity such as lymph edema, muscle wasting, neuropathy and chronic pain (Paci *et al.,* 1996). Radiation therapy is useful for cancer which is more localized, but it also carry a number of acute and chronic side- effects such as nausea, diarrhea, pain and fatigue (Ewesuedo & Ratain, 2003). Endocrine therapy may be used as a supplementary treatment. This method of therapy is applied to specific group of patients, e.g. women after menopause with hormone-responsive disease (Gradishar, 2005). In hormone-sensitive cancer patients receive chemotherapy with cytotoxic drugs. The cytotoxic drugs treat cancers by causing cell death or growth arrest. Effective cancer chemotherapy is able either to shrink a tumor or to help destroy cancer cells (Ewesuedo & Ratain, 2003). A number of obstacles such as drug toxicity, possible undesirable drug interactions and various forms of drug resistance have to be overcome to achieve effective chemotherapy (Cardosa *et al.,* 2009). Drug resistance is a general problem in the chemotherapy of several cancers including breast cancers (Wong *et al.,* 2006). Failures in treatment of cancers are common. Development of new drugs is also slow to progress. Among the reasons contributing to this are weak absorption, high rate of metabolism and elimination of drugs per oral administration resulting in less or variable concentrations in blood, poor drug solubility, unpredictable bioavailability of oral drugs due to food, and tissue toxicity (Sipos *et al.,* 1997). Thus alternative methods of drug administration like appropriate drug carrier system is needed to overcome this problem. Depending on the route of administration, the size of

drug carriers may range from a few nanometers (colloidal carriers), to micrometers (microparticles) and to several millimeters (implants). Among these carriers, nanoparticles had shown great promise for parenteral application of chemotherapeutic drugs (Mehnert *et al.,* 2001). Targeting of unhealthy tissues and organs of the body is one of the important challenges of the drug delivery systems. Nanoparticles seem to show promise as a drug targeting systems supplying drug to target tissues at the right time (Kayser *et al.,* 2005). The main objective of new drug delivery systems is to improve the anti-tumor efficacy of drug and reduce their toxic effects on normal tissues. Nanoparticle is expected to be able to diminish toxicity of chemotherapy drug. Nanoparticles based on lipids that are solid at room temperature, namely solid lipid nanoparticle (SLN) using physiological well-tolerable lipids have potentially wide application (Siekmann & Westesen, 1992; Müller *et al.,* 1995; Müller *et al.,* 2000). The SLN is a drug delivery system that loads lipophilic or chemically unstable drugs (Fig. 1). Among the advantages of SLN are high potential for management of drug release and drug targeting, high stability for drug loading and high capacity for drug payload. This delivery system makes possible the encapsulation of lipophilic and hydrophilic drugs without the toxic effect of the carriers. This system also avoids the use

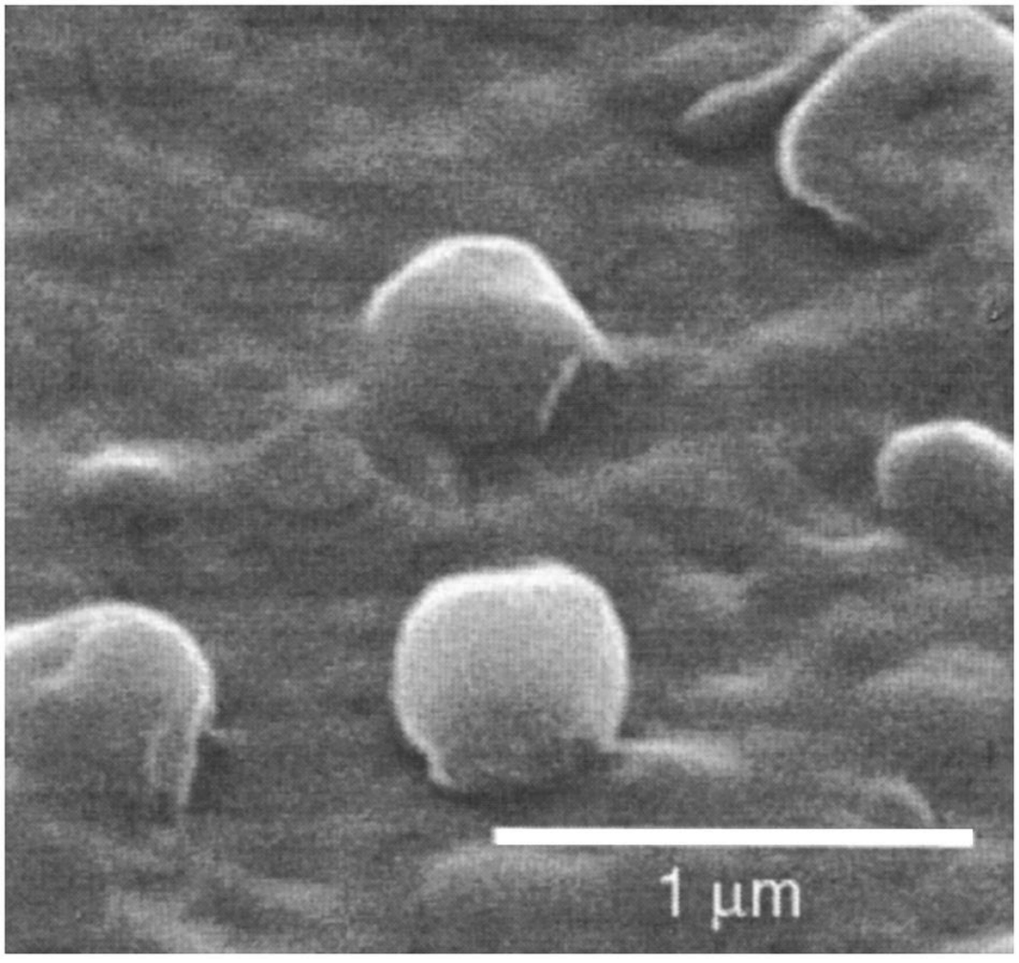

Fig. 1. Electron microscopy picture of solid lipid nanoparticles made from Compritol® stabilized with poloxamer 188, diameter 400nm (Adapted from Müller and Mäder 2000)

organic solvents, and has potential for large scale production. However, several disadvantages are associated with SLNs to include particle growth, particle aggregation, unpredictable gelation tendency, polymorphic transitions, burst drug release and inherently low incorporation capacities due to the crystalline structure of the solid lipid (Mehnert & Mäder, 2001).

The most common production technique of SLNs are high-pressure homogenization (HPH), high-shear homogenization combined with ultrasound, solvent emulsification/evaporation and microemulsion techniques. HPH is the predominant production method because it is easy to handle and scale-up. In this method, drug incorporation is achieved by dissolving or dispersing the drug in the melted lipid (He *et al.,* 2007). The drug can be encapsulated in the matrix or attached to the particle surface. In spite of improved researches in production of high quality SLN, it is still not routinely used clinically.

2. Tamoxifen-encapsulated solid lipid nanoparticles

The chemical name of tamoxifen is trans-2-[4-(1,2-diphenyl-1-butenyl)phenoxyl] N,Ndimethylethylamine (Fig. 2). Tamoxifen, an antiestrogen molecule and strong hydrophobic drug (water solubility, 0.04 μg/mL at 37°C), is widely administered in breast cancer and high risk patients (McGregor & Jorda, 1998). Although tamoxifen was primarily used as a drug against hormone-dependent breast cancers (Wyld *et al.,* 1998), it has also been used in the treatment of hormone-insensitive estrogen receptor-negative breast cancers (Jordan, 1994). Tamoxifen inhibits cell proliferation and induces apoptosis in breast cancer cells (MCF-7, MDA-MB231 and BT-20) (Mandlekar & Kong, 2001; Mandlekar *et al.,* 2000). In spite of being high effective, tamoxifen has harmful dose-dependent long-term side-effects such as development of endometrial cancer (Brigger *et al.,* 2001), hyperplasia, polyps, carcinoma, sarcoma (Peters-Engl *et al.,* 1999; Cohen, 2004) vaginal hemorrhage, blazes and liquid retention in postmenopausal breast cancer patients (Mourits *et al.,* 2001; Delima *et al.,* 2003). Formulations with the encapsulation of low-dose tamoxifen in colloidal delivery systems have been effective. Tamoxifen has been formulated in nanospheres such as poly-ε-caprolactone nanoparticles (Chawla & Amiji, 2003) and long circulating Poly(MePEGcyanoacrylate-co-hexadecylcyanoacrylate) nanoparticles in the form of free base (Brigger, *et al.,* 2001). Tamoxifen, as a nonsteroidal antiestrogen drug was recently encapsulated in SLNs and was shown to be effective on induced mammary tumor gland in Sprague-Dawley rats (Abbasalipourkabir *et al.,* 2010,1) in parenteral administration. The SLN

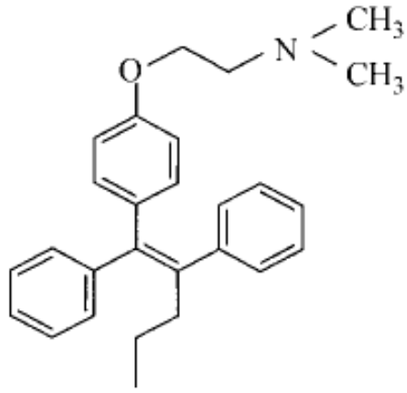

Fig. 2. Chemical Structure of Tamoxifen (Adapted from Christov *et al*., 2007)

systems offer a sustained release of the drug in its intact form (Fontana *et al.*, 2005 Using human breast cancer cell line, MCF7, some *in vitro* studies have shown that drug release from the tamoxifen-incorporated SLN has the same antitumoral activity as the free drug (Abbasalipourkabir *et al.*, 2011). Therefore the tamoxifen-loaded SLN as a carrier system has excellent potential in prolonged drug release in breast cancer therapy (Fundaro *et al.*, 2000).

2.1 Preparation of tamoxifen-loaded SLN

Drug-loaded SLNs can be prepared using the high-pressure homogenization technique (Abbasalipourkabir *et al.*, 2011). A mixture of Hydrogenated palm oil (Softisan 154 or S154) and Hydrogenated soybean lecithin (Lipoid S100-3, containing 90% phosphatidylcholine, including 12–16% palmitic acid, 83–88% stearic acid, oleic acid and isomers, and linoleic acid] at a ratio of 70:30 is grounded in a ceramic crucible. The mixture is heated to 65–70°C while being stirred with a PTFE-coated magnet until a clear-yellowish lipid matrix (LM) solution is obtained. A solution containing 1 mL oleyl alcohol, 0.005 g thimerosal, 4.75 g Sorbitol, and 89.25 mL bidistilled water (all w/w) at the same temperature is added to 5 g of LM. A pre-emulsion of SLN is obtained using the homogenizer (Ultra Turrax, Ika) at 13,000 rpm for 10 min and high-pressure homogenizer (EmulsiFlex-C50 CSA10, Avestin) at 1000 bar, 20 cycles, and 60 °C. The lipophilic drug tamoxifen (1 mg) is dissolved in oleyl alcohol and mixed with 5 mg of SLN pre-emulsion using the Ultra Turrax homogenizer at 13,000 rpm for 10 min. This mixture is then incubated overnight at 50–60 °C, stirred periodically with a PTFE-coated magnet at 500 rpm, and finally will expose to air to solidify.

2.2 *In Vitro* antitumoral activity of Tamoxifen-loaded solid lipid nanoparticle

Cell death basically can occur in two ways. The first is through the necrosis pathway, where traumatic injuries cause cells damage in particular cell enlarges, bursts and liberate its intracellular components into the surrounding environment. The second pathway is programmed cell death or apoptosis, which is a molecular signaling cascade, inducing a disturbance in the organization and package of the cell causing death (Fadok, 1999; Messmer & Pfeilschifter, 2000). Other mode of cell death has also been suggested, for example mitotic cell death, which plays an important role in cell death caused by ionizing radiation (Steel, 2001). Breast cancer is the most common malignancy (18% of all malignancies) in women worldwide and its occurrence is slowly increasing (Salami & Karami-Tehrani, 2003). Like many cancers, breast cancer appears to be a result of high genetic damage that caused uncontrolled cellular proliferation and unusual apoptosis. These phenomena activate proto-oncogenes and inactivate tumor suppressor genes. These events can be activated by exposure of living cells to environmental, physical, chemical and/or biological carcinogens (Russo & Russo, 2002). The antiestrogen molecule, Tamoxifen (TAM) or trans-2-[4-(1,2-diphenyl-1-butenyl) phenoxyl]-N, N-dimethylethylamine, has been widely applied in treatment of breast cancer and high risk patients. Tamoxifen can reduce the occurrence of contralateral breast cancers by at least 40% (Fontana *et al.*, 2005). Tamoxifen exhibits anti-estrogenic activity by binding to the intracellular estrogen receptor. The tamoxifen-estrogen receptor complex binds with DNA and can subsequent inhibit mRNA transcription and lead to cellular apoptosis (Chawla & Amiji, 2003). Recently nanoparticulate delivery systems in the form of nanospheres like poly- caprolactone

nanoparticles and long–circulating PEG-coated poly (MePEGcyanoacrylate-co-hexadecylcyanoacrylate) nanoparticles in the form of free base have been used for tamoxifen encapsulation. The basis of this formulation is to obtain the necessary dose of drug at tumor location for a known period of time and reducing adverse effects on normal organs in the body (Chawla & Amiji, 2003). In recent years Delivering Tamoxifen within Solid Lipid Nanoparticles have been recommended. Animal models play an important role in cancer chemotherapy (Abbasalipourkabir *et al.,* 2010,2). However, today there is increasing acceptation for *in vitro* tests as the method for determining cytotoxicity and viability of chemotherapeutic drugs. The reason for change lies partly in the limitations of animal models, to include financial considerations, time, and differences between animal and human metabolism. Finally, there is the moral pressure to reduce animal experimentation. *In vitro* tests are more likely to be reproducible. In general, the procedure involves the exposure of cells to a range of concentrations of the chemicals under test for a defined time and then to test for cell viability. Such tests are most easily performed in microtitre plates, which allow rapid quantitation of the results using a micrometer plate reader (Adams, 1990). The responses of breast cancer cell lines are determined by cytotoxicity assay, cellular and nuclear morphology, apoptosis and cell cycle distribution.

2.3 Cytotoxicity effect of TAM-loaded SLN on human breast cancer cells

The TAM-loaded SLN has an equally efficient cytotoxic activity as free tamoxifen. Therefore TAM-loaded SLN preserves the antitumoral activity of the free drug. When TAM is incorporated into the SLN carrier system, its antitumoral activity is still maintained and formulating TAM by incorporating into SLN will potentially enhance the solubility of the drug through inclusion into the lipid phase and facilitating the entrapment of greater amounts of the drug in the SLN, suggesting that SLN is a good carrier for the drug (Fig. 3. & Fig. 4).

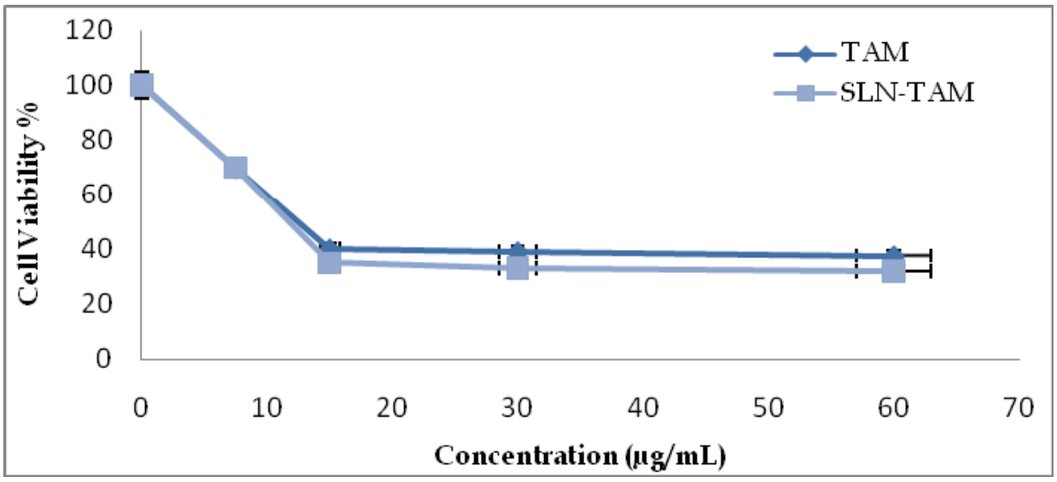

Fig. 3. MCF-7 cells viability after 72 hours incubation with TAM and TAM-Loaded SLN formulation. The percentage of cell viability is expressed as a ratio of treated cells to the untreated control cells. Each point represents the mean ± standard deviation of 5 wells. (Adapted from Abbasalipourkabir, 2010).

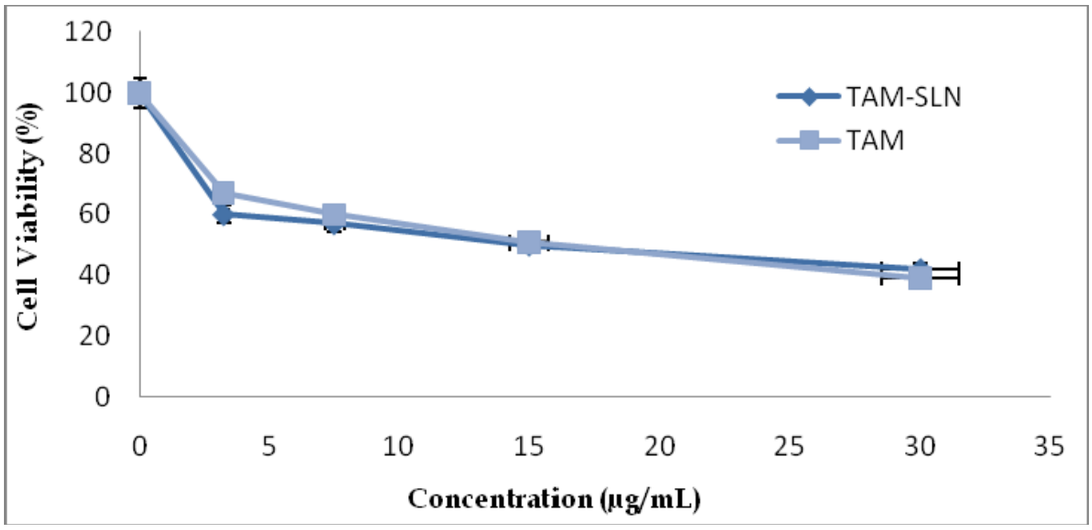

Fig. 4. MDA-MB231 cells viability after 72 hours incubation with TAM and TAM-Loaded SLN formulation. The percentage of cell viability is expressed as a ratio of treated cells to the untreated control cells. Each point represents the mean ± standard deviation of 5 wells. (Adapted from Abbasalipourkabir, 2010).

The IC_{50} of TAM and TAM-loaded SLN for MDA-MB231 cells (ER-negative or ER-independent) is higher than for MCF-7 cells (ER-positive or ER-dependent), (Tables 1-2).

Treatment	IC_{50} (µg/mL)		
	24h	48h	72h
TAM	13.45±0.46	13.00±0.98	12.50±0.91
TAM-SLN	13.18^{a}±0.66	12.50^{a}±1.50	11.78^{b}±0.18

All value represent the means ± std. dev., (n=5) ^{A,b}means in each row with different superscripts are significantly different TAM = Tamoxifen; TAM-SLN = Tamoxifen-loaded solid lipid nanoparticle (Adapted from Abbasalipourkabir, 2010).

Table 1. The IC_{50} of TAM and TAM-loaded SLN on MCF-7 cells after 24, 48 and 72h.

Treatment	IC_{50} (µg/mL)		
	24h	48h	72h
TAM	17.21±1.44	16.87±1.97	15.97±0.86
TAM-SLN	16.93±0.82	16.00±0.10	15.80±0.69

All value represent the means ± std. dev., (n=5) TAM = Tamoxifen; TAM-SLN = Tamoxifen-loaded solid lipid nanoparticle (Adapted from Abbasalipourkabir, 2010).

Table 2. The IC_{50} of TAM and TAM-loaded SLN on MDA-MB231 cells after 24, 48 and 72h.

The mechanisms of ER-independent, TAM-induced apoptosis may be through the inhibition of protein kinase C. The IC_{50} value of tamoxifen for protein kinase C inhibition is 4 to 10 times the concentration for ER inhibition in ER-positive cells. Therefore, the dose of

tamoxifen for treatment of patients with ER-positive breast cancer would have to be increased over the usual 20 mg per day used. High dose of tamoxifen might decrease the therapeutic index by increasing toxicity (Gelmann, 1996). It seems that improved cytotoxicity of incorporated drug is not dependent of the composition on the SLN. In fact it was reported that the IC_{50} value of drug-loaded SLN composed of different materials were lower than that of free drug solution (Yuan *et al*, 2008). There are at least two mechanisms that have been associated with the cytotoxicity of drug-loaded SLN. Using Doxorubicin (DOX)-loaded SLN, it was suggested that the first mechanism involves the release of DOX from DOX-SLN outside the cells, and the cytotoxicity of DOX is increased by the nanoparticles. The second mechanism suggested was, release of the drug inside the cell and thus produces greater cytotoxicity (Wong *et al*., 2006).

2.4 Morphological changes of TAM-loaded SLN on human breast cancer cells

Apoptotic cell death can be recognized under phase contrast and fluorescence inverted microscope after staining. This is the most practical method to identify cell morphological changes attributed to apoptotic cell death.

2.4.1 Phase contrast microscopy

TAM-loaded SLN treatments at concentrations equal to IC_{50}, causes detachment of MCF-7 and MDA-MB231 cells and loss of colony formation ability. These cells appear rounded-up and lose contact with neighboring cells (Fig. 5). The normal untreated MCF-7 and MDA-MB-231 cells, however, appear healthy and exhibiting epithelial–like features and forming a monolayer on the surface of the culture flask. In the presence of TAM and TAM-loaded SLN, the viability of the both cells diminishes and the cancer cells loss their normal morphological characteristics, detaches, aggregates, and later develops apoptotic bodies. The detachment of cells in the presence of free TAM and TAM-loaded SLN suggests that tamoxifen is cytotoxic, even when incorporated in the SLN.

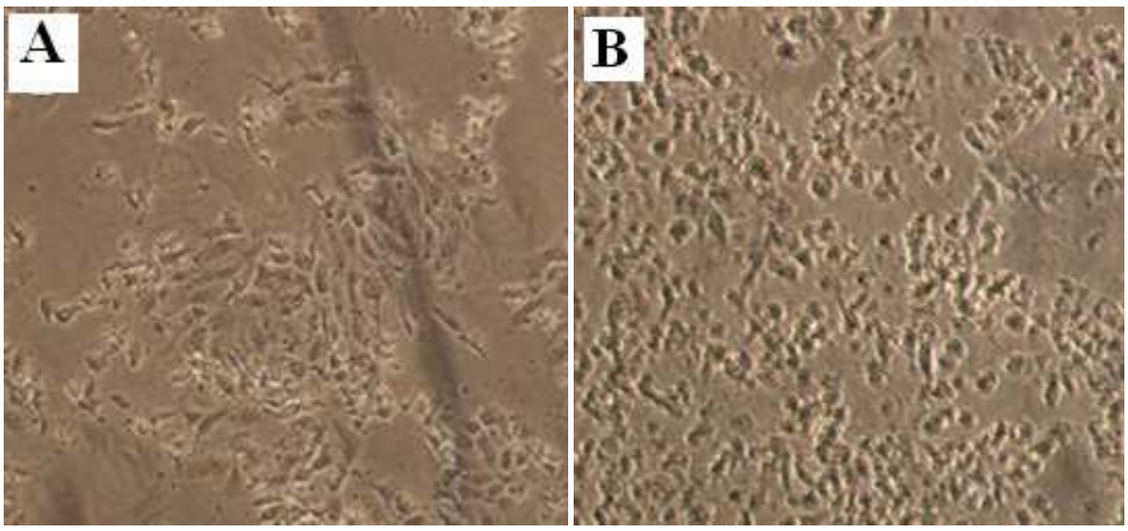

Fig. 5. Phase contrast micrographs of MCF-7 cell (A) and MDA-MB231 cell (B) treated with TAM loaded SLN (12 µg/mL) (magnification x10). (Adapted from Abbasalipourkabir, 2010).

2.4.2 Nuclear morphology

Cell death is either by physiological or pathological means. Physiological cell death is distinguished by apoptotic morphology, including chromatin condensation, membrane blebbing, internucleosomal degradation of DNA, and apoptotic body formation. Pathological cell death or necrosis is associated with cellular swelling and collapse, without severe damage to nuclei or breakdown of the DNA. In apoptosis, several cellular and molecular biological features, including cell shrinkage and DNA fragmentation are exhibited (Yu *et al.*, 2010). To characterize the cell death, the nuclear morphology of dying cells can be examined under Hoechst dye 33258 staining. The Hoechst dye 33258 is a bis-benzimide derivatives and a fluorescent DNA-binding agent. This dye is useful for cell cycle analysis because it can be used in low concentrations, and thus minimizing the problem of toxicity. According to Latt & Stetten, (1976) the Hoechst dye binds to AT-rich regions of the DNA and when excited with an ultraviolet light produces bright fluorescence at 465 nm. TAM-loaded SLN induce death of MCF-7 and MDA-MB231 cells by apoptosis. This is evident by the typical apoptotic changes showing clear condensation of cell nuclei, nuclear fragmentation and apoptotic bodies (Fig. 6).

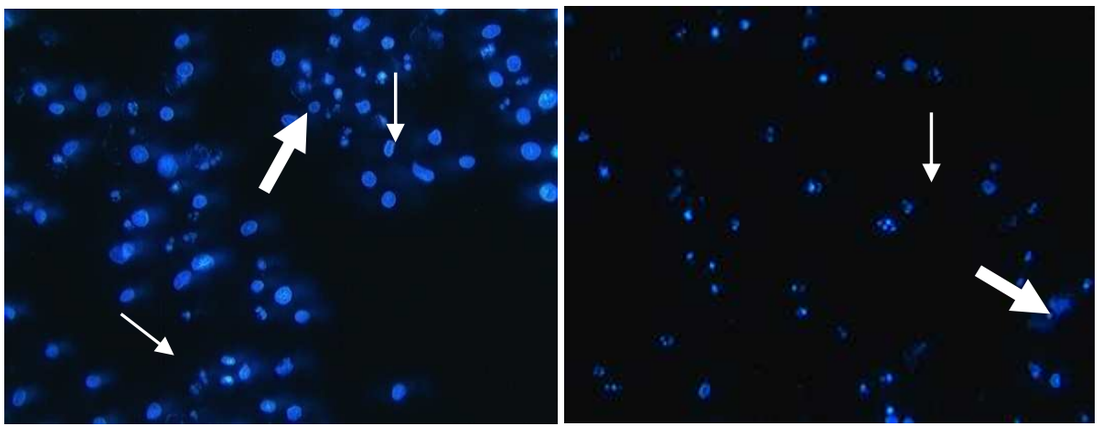

Fig. 6. Fluorescence microscopy of MCF-7 cells (A) and MDA-MB-231 cells (B) treated with TAM-loaded SLN. Cell shrinkage (thin arrow) and apoptotic cells (thick arrow) are evident (magnification x40). (Adapted from Abbasalipourkabir, 2010).

Tamoxifen-loaded SLN like free TAM display antitumoral activity against human breast cancer cells. The biological availability of drug is not affected when incorporated into SLN. Therefore SLN could be applied as a drug delivery system for cancer treatments. In conclusion, the TAM-loaded SLN, because of its small size, could not be easily phagocytosed by macrophages and therefore the nanoparticles could be potentially used in long-term circulating carrier system for breast cancer therapy.

3. Conclusion

The main challenge in cancer chemotherapy is toxic side-effects induced by chemotherapeutic drugs. Single dose or short-time application (1-2 weeks) will probably causes serious health problems, but the use of biodegradable nano-sized particles for

long-term or life-time therapy may produce other serious side-effects. Increasing the encapsulation efficiency of poorly water-soluble molecules will lead to the development of improved SLN formulations. In the near future, it is expected more studies will focus on improving SLN and drug-loaded SLN formulations to increase the efficacy and reduce the side-effects of chemotherapeutic drugs for anticancer treatment. These studies should include preparation of formulations with different particle size and distributions, different matrix lipids and additional ingredients. Thus, if nanoparticulate drug delivery systems to be used effectively and routinely, the matter of toxicity of the components of nanoparticles must be addressed. Indeed, SLN requires further development before it can be used as a new drug delivery system for chemotherapy drugs in treatment of human cancers.

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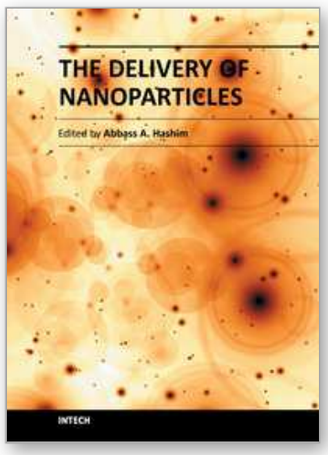

The Delivery of Nanoparticles
Edited by Dr. Abbass A. Hashim

ISBN 978-953-51-0615-9
Hard cover, 540 pages
Publisher InTech
Published online 16, May, 2012
Published in print edition May, 2012

Nanoparticle is a general challenge for today's technology and the near future observations of science. Nanoparticles cover mostly all types of sciences and manufacturing technologies. The properties of this particle are flying over today scientific barriers and have passed the limitations of conventional sciences. This is the reason why nanoparticles have been evaluated for the use in many fields. InTech publisher and the contributing authors of this book in nanoparticles are all overconfident to invite all scientists to read this new book. The book's potential was held until it was approached by the art of exploring the most advanced research in the field of nano-scale particles, preparation techniques and the way of reaching their destination. 25 reputable chapters were framed in this book and there were alienated into four altered sections; Toxic Nanoparticles, Drug Nanoparticles, Biological Activities and Nano-Technology.

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